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Neuronal-glia populations form functional networks in a biocompatible 3D scaffold

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Abstract

Monolayers of neurons and glia have been employed for decades as tools for the study of cellular physiology and as the basis for a variety of standard toxicological assays. A variety of three dimensional (3D) culture techniques have been developed with the aim to produce cultures that recapitulate desirable features of intact. In this study, we investigated the effect of preparing primary mouse mixed neuron and glial cultures in the inert 3D scaffold, Alvetex. Using planar multielectrode arrays, we compared the spontaneous bioelectrical activity exhibited by neuroglial networks grown in the scaffold with that seen in the same cells prepared as conventional monolayer cultures. Two dimensional (monolayer; 2D) cultures exhibited a significantly higher spike firing rate than that seen in 3D cultures although no difference was seen in total signal power (<50Hz) while pharmacological responsiveness of each culture type to antagonism of GABA_AR, NMDAR and AMPAR was highly comparable. Interestingly, correlation of burst events, spike firing and total signal power (<50Hz) revealed that local field potential events were associated with action potential driven bursts as was the case for 2D cultures. Moreover, glial morphology was more physiologically normal in 3D cultures. These results show that 3D culture in inert scaffolds represents a more physiologically normal preparation which has advantages for physiological, pharmacological, toxicological and drug development studies, particularly given the extensive use of such preparations in high throughput and high content systems.

Keywords: Cortical cultures, microelectrode array, biocompatible scaffold, 3D neuronal culture, murine

Introduction

Monolayer cultures of murine primary neurons form spontaneously active networks *in vitro*, and are commonly used in pharmacological, toxicological and electrophysiological studies [3, 4]. In this regard, microelectrode arrays (MEAs) are particularly well suited to physiological, pharmacological and toxicological studies [4, 5, 16] of such cultures since they permit non-invasive, long term (weeks to months) monitoring of developmental and treatment-induced changes in neuronal network function [5]. However, conventional monolayer cultures do not reliably reproduce some physiological features of central nervous system tissue *in vivo* or, as acute brain slices *in vitro/ex vivo* in particular synchronous large amplitude local field potentials are absent [5]. This limitation can be addressed in part via organotypic slice cultures, but is further complicated by requirements for a media interface and the anatomical changes that are found in slice cultures within a few days *in vitro*.

In order to exploit the potential of MEAs but address the limitations described above, it has been speculated that three dimensional (3D) cell culture approaches could be used. To this end, numerous substrates have been successfully developed which are largely gel-based and contain extracellular matrix (ECM) components to influence cell development [1]. The ideal 3D matrix is biocompatible, persistent, easily manipulated (i.e. non-gel), can be coated with ECM components as required, seeded with defined cells and either grown *in situ* on an MEA or independently before assay using an MEA (i.e. comparable to use of acute brain slices [7]).

Alvetex 3D cell culture scaffold [6] is commercially available, polystyrene-based and of 200µm thickness (Figure 1). The scaffold contains voids of variable sizes in which seeded cells can develop and can be shaped to fit MEAs and easily moved from culture vessels to MEAs. Additionally, small Alvetex circles (~6mm diameter) require fewer seeded cells than

a monolayer seeded MEA (~40 mm diameter); an important consideration for high cost cultures (e.g. stem cells [13]) and in the reduction of animal use in research [9]. **Related scaffolds have been used to support neuronal-glia co-culture in a peripheral nerve preparation [2] but a viable and functional central nervous system preparation is lacking.**

Here, we investigate and characterise functional murine neuronal networks cultured in Alvetex by using MEA and immunocytochemical approaches to demonstrate their relevance, efficiency and economy as viable and attractive tissue culture models. **We compare the electrophysiological features of monolayer and 3D cultures in addition to their pharmacological responsiveness. We present immunocytochemical findings to reveal morphological differences in astroglial cells under different conditions and differences in bioelectrical activity suggesting a more physiologically normal profile for 3D cultures.**

Materials & Methods

Chemicals were from Sigma Aldrich. Tissue culture media, supplements, secondary antibodies and immunochemistry reagents were from Life Technologies (Invitrogen) unless otherwise stated. Alvetex was supplied by Reinnervate (Co. Durham, UK) and a single-hole punch used to make 6mm diameter circles which were placed in a 48 well-plate and washes of 5 minutes each in 70% ethanol, Dulbecco's phosphate buffered saline (PBS; twice; **pH7.4**) and DMEM. 0.3mL DMEM+10% fetal bovine serum (FBS) was added to each well and plates equilibrated at 37°C 5% CO₂ overnight. MEAs and coverslips for immunocytochemistry were coated with 25µg/cm² poly-D-lysine in deionised water for 5 minutes and dried for 2 hours. 1mL (MEAs) or 0.5 mL (coverslips) DMEM+10% FBS was added and equilibrated at 37°C 5% CO₂ overnight.

Embryonic mouse cortical neuronal cultures: Primary embryonic mouse cortical neuroglial cultures were based on [15]. E14 embryos were obtained from timed mated NIHS mice in compliance with the UK Animals (Scientific Procedures) Act, 1986, decapitated, brains removed, cortices dissected and meninges removed in Dulbecco's PBS minus calcium and magnesium plus 33mM glucose (PBS-G). Cortices were mechanically dissociated in 10ml PBS-G, allowed to settle for 5 min before the supernatant was decanted and centrifuged (200g; 5 min) at room temperature. The resulting cell pellet was resuspended in 10ml DMEM:F12 (1:1) with: L-glutamine 2mM, HEPES 5mM (**pH7.4**), glucose 33mM, 6.5mM NaHCO₃, 100IU/ml penicillin-streptomycin, supplemented with: insulin 25µg/ml, transferrin 100µg/ml, putrescine 60 µg/ml, progesterone 20nM and Na₂O₄Se 30nM. Resuspended cells were counted using trypan blue dye exclusion and diluted to 1x10⁶ viable cells/ml in media consisting of MEM supplemented with 5% heat inactivated horse serum, L-glutamine 0.5mM, glucose 15mM and gentamicin sulfate 10µg/ml (neuronal medium). Cell suspension

was diluted in neuronal medium and added to MEA dishes and multi-well plates at $\sim 2.5 \times 10^5$ cells/cm² of culture surface. 50% of medium was replaced with fresh neuronal medium and on DIV 3, 5, 7, 10, 12, 14 and every other day thereafter for the life of the culture. Monolayer cultures on MEAs were sealed using plastic rings holding a 12.5µm thick Teflon semi-permeable membrane to prevent evaporation but allow gas exchange. All results reported were obtained from cultures at DIV14-21, consistent with previous reports showing the establishment of complex neuroglial networks that exhibit robust bioelectrical activity at this stage of development [16].

Immunocytochemistry: Cells on coverslips and Alvetex were fixed by transfer to 24 well plates containing 0.5mL/well PBS, three brief washes with 0.5mL PBS and 10 minute incubation with 0.5mL PBS containing 3.7% formaldehyde. Wells were then washed in PBS (thrice) and 0.5ml PBS+0.02% Triton-X100 added for 2 minutes before washing with PBS+5% FBS (thrice) and incubation 2 hours with 0.5mL of PBS+10% goat serum. Serum solution was aspirated and 0.5ml primary antibody diluted in PBS, or PBS alone (control), added to wells overnight at 4°C. All wells were then washed with 0.5mL PBS+5% FBS (thrice) for 2 minutes. 0.5ml fluorophore-conjugated goat anti-rabbit or anti-mouse secondary antibody in PBS was added to all wells and incubated for 2 hours at room temperature before washing with 0.5mL PBS (thrice) for 2 minutes. 0.5mL 10 µg/ml Hoechst 33342 blue solution was added for 5 seconds before flooding with PBS and brief rinsing and draining with 0.5mL PBS (thrice). Coverslips and Alvetex were removed from wells, mounted on slides using fluorescence mounting medium (Vector Laboratories) and sealed using nail varnish before visualisation using epifluorescence microscopy. Primary antibodies (supplier and dilution) used were: GFAP (Dako, 1:1000), β-Tubulin III (Millipore, 1:1000). AlexaFluor-conjugated secondary antibodies (Invitrogen) were used at 1:500 dilution.

MEA Recordings: Planar 60 electrode (30µm diameter; 200µm spacing) MEAs

(MultiChannelSystems, Reutlingen, Germany) were used. MEAs were placed on a headstage maintained at 37°C and signals amplified (1200× gain) by a 60-channel amplifier (MEA60 System, MultiChannelSystems) and simultaneously sampled at 25kHz/channel. Data were acquired to and analysed using a PC with MCRack (MultiChannelSystems). Offline bandpass filtering (50Hz), spike detection (-5.5 standard deviations from root mean square of the noise level) and analysis were performed using MC_Rack and Neuroexplorer 4 (Nex Technologies, USA) software using 300s recordings taken at steady states where bursts were detected via interval specification algorithm (**200Hz high pass filter**; burst max start/end: 10ms; min interval between bursts: 10ms; min burst duration: 20ms; min spikes/burst: 4). Media in 2D MEA cultures was replaced with extracellular recording solution (ECS) immediately prior to experiments. For recordings from cultures in Alvetex, MEA dishes maintained at 37°C 5% CO₂ overnight filled with 1ml DMEM+ 10% FBS then briefly washed with 1mL ECS before Alvetex circles were placed directly over the MEA active area weighed down with 0.3-0.5cm diameter silver rings before addition of 0.25mL ECS. ECS contained 140mM NaCl, 5mM KCl, 2mM CaCl₂, 10mM HEPES, 10mM glucose, 2µM glycine, 2mM MgCl₂ (pH 7.3). All cultures were equilibrated on the MEA headstage for 15 minutes prior to recordings. BMI (GABA_A receptor antagonist), TTX (voltage-gated sodium channel blocker), CNQX (AMPA/kainate receptor antagonist) and DL-AP5 (NMDA receptor antagonist; Abcam, Bristol, UK) were prepared as stock solutions in water or dimethylsulfoxide and stored as single use aliquots at -20°C. Before use, aliquots were thawed and appropriate volumes of stock solution added to ECS in the MEA to provide the correct final concentration.

Statistics: Comparisons between different culture types were undertaken using either Wilcoxon signed rank tests in the case of comparisons of normalized data or unpaired Student's t-tests in the case of absolute values. In all cases, statistical significance was accepted when $P < 0.05$. Correlation between measures at individual electrodes were determined by Spearman's rank correlation metric in Matlab. Where data were obtained from multiple MEA channels, results per channel were pooled to provide values per MEA that were treated as individual replicates for statistical purposes.

Results

Immunocytochemistry: In both 2D (Fig. 2A) and 3D (Fig. 2B) cultures, positive staining for GFAP and β -tubulin III was seen, representing glial (astrocyte) and neuronal cell populations respectively. GFAP-positive cell morphology showing extensive and intricate processes in 3D was consistent with that of astrocytes in intact mouse brain slices [10] and was in contrast to morphologically planar GFAP-positive cells with short processes seen in 2D cultures [8]. These results confirm the presence of viable neuronal and astrocyte cell populations in both 2D and 3D cultures in addition to suggesting a substrate-induced morphological difference in astrocyte populations.

MEA Recording: Spontaneous action potential firing (Fig. 3A&B) overlying limited local field potential events (Fig. C) were detected in both 2D and 3D cultures. In control conditions, spike firing rate was significantly lower in 3D cultures than in 2D cultures ($p < 0.0001$; unpaired t-test; $n = 6$ per culture type; Fig. 3A) although no significant difference in mean total signal power $< 50\text{Hz}$ was seen between cultures ($P > 0.05$; unpaired T-test; $n = 6$ per culture type; Fig. 3C&D).

In both culture types, spike firing was significantly increased by the GABA_A antagonist, BMI ($20\mu\text{M}$; $p < 0.05$ in each; $n = 6$ per culture type) indicating intact GABAergic transmission (Fig. 3E&F). Subsequent treatment with the glutamate receptor antagonists CNQX ($20\mu\text{M}$; AMPA/kainate) plus DL-AP5 ($100\mu\text{M}$; NMDA) significantly reduced normalized spike firing rate ($P < 0.05$ vs control for each; $n = 6$ per culture type) indicating intact glutamatergic transmission (Fig. 3E&F) while, finally, treatment TTX ($1\mu\text{M}$) also significantly reduced firing rate vs control (Fig. 3E&F). In order to further probe the relationship between action potential firing and local field potential events in 2D and 3D systems, we investigated the level of correlation between results obtained for the specific

measures previously described. In control and BMI treatment conditions, a very strong positive correlation (shown hereafter as Spearman's r) was seen between spike firing and burst incidence in both 2D (CTL: 1; BMI: 1) and 3D (CTL: 0.97; BMI: 1) suggesting the expected interdependence between these phenomena wherein the majority of spikes occur during bursts. Interestingly, in control cultures, while a small positive correlation between burst incidence and total signal power (<50Hz) was seen in 2D (0.38), a negative correlation was observed in 3D cultures (-0.5) suggesting that, unlike 2D, in physiologically normal conditions in 3D, local field potential activity is not associated with spike bursts. When 3D cultures were disinhibited via treatment with BMI, the correlation between burst incidence and total signal power (<50Hz) became positive (0.48) suggesting that local field potential events now associated with spike bursts as was previously seen in 2D cultures in control conditions. In BMI, the correlation between burst incidence and total signal power (<50Hz) **in 2D cultures** was largely unchanged (0.32).

Discussion

While a variety of commercial and bespoke scaffolds which support 3D cell culture are now in use [11] and neuronal-glial cultures have previously been grown on Alvetex membranes [14], our results demonstrate for the first time that spontaneously active, functional networks can be formed and monitored using murine central nervous system cells grown within this substrate. **Specifically, we show viable murine mixed neuronal-glial cultures grow in 3D within the Alvetex substrates.** Furthermore astrocyte morphology is notably different in such 3D cultures when compared with 2D monolayers. This is particularly interesting when the astrocyte morphology seen in 3D culture (extensive, intricate processes) was more consistent with astrocyte morphology *in vivo* than with that seen in comparator 2D monolayer cultures, as previously described [14]. **However, given growing recognition of the ‘quadpartite synapse’ [12], investigations of microglia in 3D scaffolds such as Alvetex are warranted.**

In addition to the more physiologically normal features of astrocytes present in these mixed neuroglial cultures maintained in 3D, robust spike firing activity was also seen. This activity could be reliably and predictably modulated with pharmacological agents, consistent with the responses seen in conventional 2D systems. The results demonstrate that both GABAergic and glutamatergic transmission are active in 3D neuroglial networks. The mean spike firing rate observed in 3D cultures was significantly lower than that seen in 2D cultures although the immunostaining of neurons in 3D cultures shows that this lower rate is not a result of a loss of viability. We propose that the lower firing rate **may arise from** a greater distance between cells and MEA electrodes when 3D cultures grown in Alvetex are placed on an MEA, compared to 2D cultures grown directly on an MEA, thereby attenuating low amplitude individual action potential events. Alternatively or in addition to signal attenuation, since both monolayer cultures and 3D scaffolds were seeded with the same cell suspension

and density calculated by area and not by volume, it is likely that some viable neurons within 3D scaffolds lay outside of the MEA electrode recording horizon since 30µm MEA electrodes typically detect signals within a 100µm radius yet the Alvetex membrane is 200µm in thickness.

Despite the significantly lower spike firing rate seen in 3D cultures, no difference in signal power at frequencies <50Hz (reflecting local field potential and volume conduction events) between 2D and 3D systems was seen. However, when the relationship between spike firing, burst events and low frequency signal power were investigated further, it was clear that burst events drive the majority of local field potential activity in 2D monolayer cultures whereas, in 3D cultures, low frequency local field potentials occur more frequently outside burst events in control conditions but become more strongly associated with bursting when disinhibited. This response from 3D cultures is more similar to the behavior of acute slice preparations [7] than the comparator 2D cultures and so highlight a unique advantage of 3D culture.

Taken together, our results show that primary, murine neuroglial cultures can be successfully seeded in inert 3D scaffolds to produce more physiologically normal networks that exhibit spontaneous bioelectrical activity with features more closely reminiscent of conventional acute brain slice preparations than confluent monolayer cultures of the same cells. This approach represents a step forwards in the use of more physiologically relevant cell culture techniques that are of value in pharmacological and toxicological studies associated with drug development in addition to **analyses** studying cellular physiology of neuronal networks. More relevant models are likely to reduce the likelihood of false negative or positive results in commonly used high throughput and high content approaches.

Figure legends

Figure 1: Scanning electron micrograph of Alvetex 3D scaffold in transverse section. The material is 90% porous and comprised of voids (36-40µm diameter) with interconnecting pores (13-15µm diameter) engineered into a 200µm thickness membrane.

Figure 2: Immunocytochemical staining of DIV14 (**A**) 2D and (**B**) 3D cell cultures, showing the neuronal marker β -Tubulin III (AlexaFluor 488; **blue**), the glial marker GFAP (AlexaFluor 633; **green**), nuclei (Hoechst 33342; **red**) and an overlay of stains in each culture. Bottom panels in each of **A** and **B** show an enlarged image of GFAP staining in each culture type to highlight morphological differences observed.

Figure 3: MEA recordings from 2D and 3D cultures. (**A**) Representative 60 channel MEA recordings from 2D (upper panel) and 3D (lower panel) cultures. (**B**) Comparison of mean spike firing frequency between 2D (**gray**; n=6) and 3D (**black**; n=6) cultures. **Insets show 500 spike overlay (light gray) and overage spike waveform (white) Scale: 50µV, 5ms** (**C**) Comparison of mean local field potential power (frequencies <50Hz) between 2D (**gray**; n=6) and 3D (**black**; n=6) cultures where (**D**) shows composite (n=6 per condition) power spectral density profiles for 2D (**gray**) and 3D (**black**) cultures. Effects of neurotransmitter receptor antagonists on spike firing in 2D (**E**) and 3D (**F**) cultures.

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Figure 1)

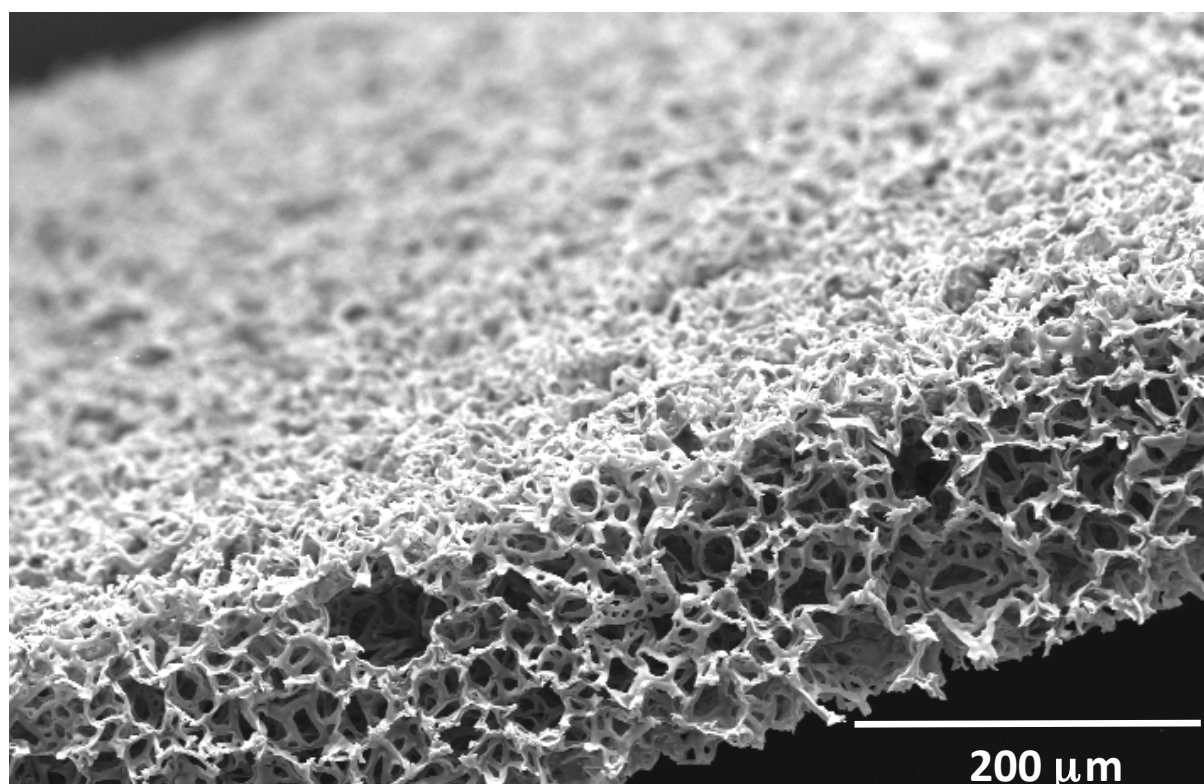
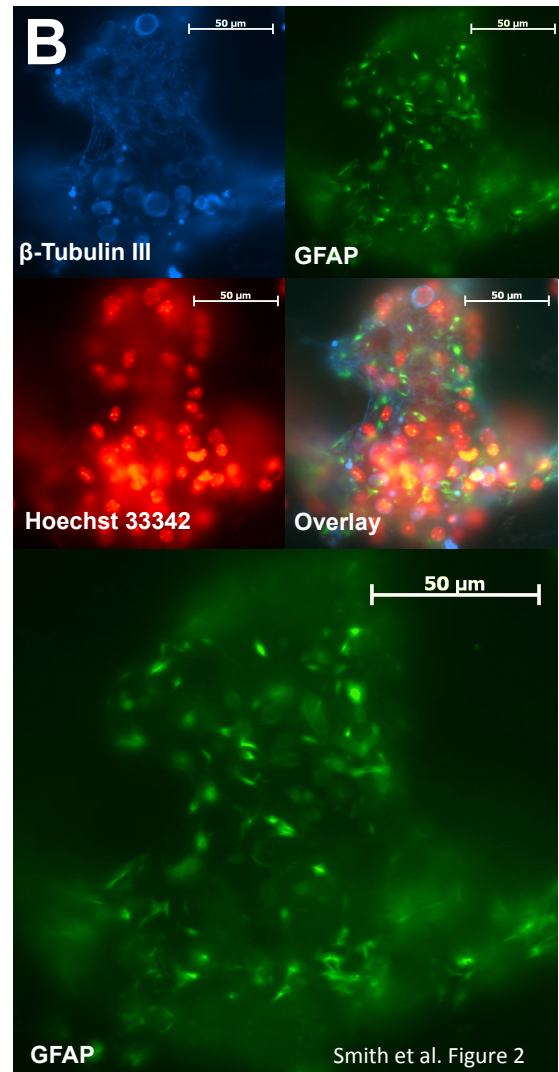
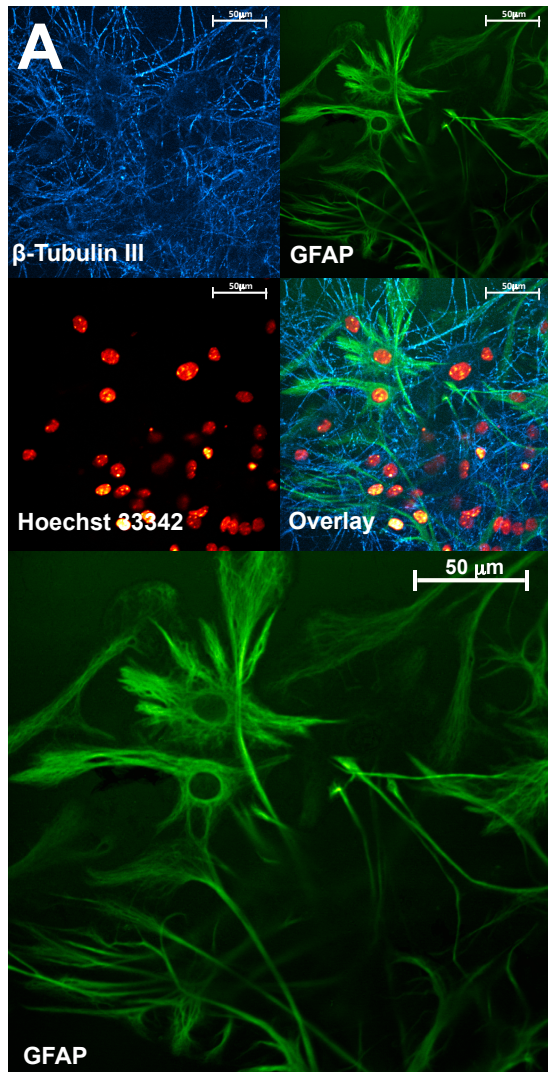
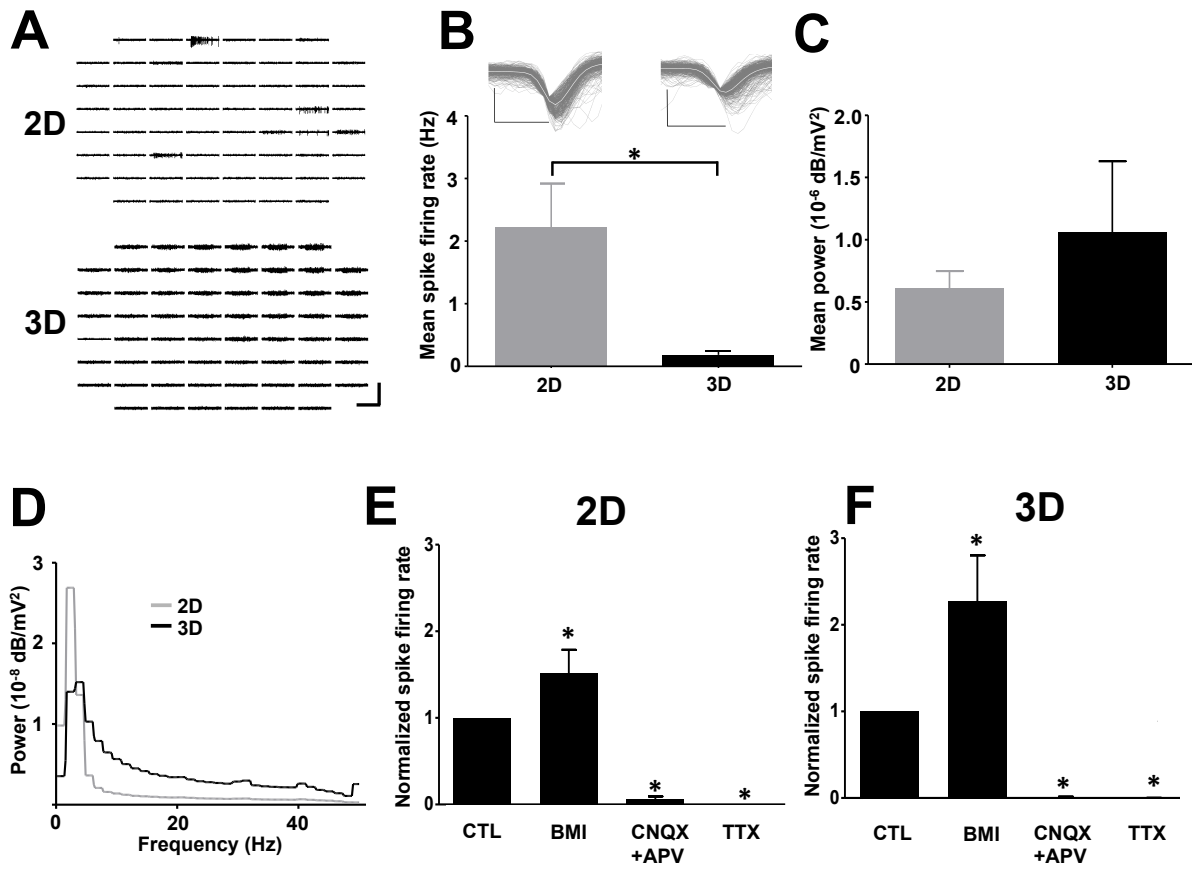


Figure 2)



Smith et al. Figure 2

Figure 3)



Smith et al. Figure 3